Faculty of Resource Science and Technology

THE EFFECT OF DIFFERENT TYPES OF INOCULUM ON DIFFERENT TYPES OF SUBSTRATES ON FERULIC ACID PRODUCTION BY *Ceratocystis paradoxa* for BIOVANILLIN PRODUCTION

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The Effect of Different Types of Inoculum on Different Types of Substrates on Ferulic Acid Production by *Ceratocystis paradoxa* for Biovanillin Production

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DECLARATION

I hereby declare that this Final Year Project report 2015 entiltled "The Effect of Different Types of Inoculum on Different Types of Substrates on Ferulic Acid Production by *Ceratocystis paradoxa* for Biovanillin Production" is based on my original work except for the quotation and citations which have been dully acknowledged. I also declared that it has not been or concurrently submitted for any degree at UNIMAS or other instituitions of higher learning.

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LIST OF ABBREVIATIONS

FA	=	Ferulic acid
LCW	=	Lignocellulose wastes
MSM	=	Minimal Salt Medium
Spore/ml	=	Spore per millimeter
SLF	=	Submerged Liquid Fermentation
SSF	=	Solid State Fermentation
g/l	=	Gram per liter
mg	=	Milligram
mg/ml	=	Milligram per millimeter
MeOH	=	Methanol
dH_2O	=	Distilled Water
nm	=	Nanometer
µl	=	Micro liter
µm	=	Micrometer
°C	=	Degree Celcius

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The Effect of Different Types of Inoculum on Different Types of Substrates on Ferulic Acid Production by *Ceratocystis paradoxa* for Biovanillin Production

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ABSTRACT

This research was done to compare the utilization of pineapple peel and banana peel as substrate for microbial production of ferulic acid-the precursors of vanillin by *Ceratocystis paradoxa* under solid state fermentation (SSF). This study was vital as no others research had reported any positive production from *Ceratocystis paradoxa* as this fungus was known as polyphagous wound parasite causing black rot post-harvest disease in pineapple and yet there was no study about solid state fermentation of *Ceratocystis paradoxa* previously. Apart from that, this researched also aimed to investigate the difference between two different type of inocula; spore suspension and solid mycelium plug used in the fermentation in producing high titre of ferulic acid. From the result, pineapple peel gives the highest ferulic acid production of 5.3885 mg/g compared to banana peel which has the maximum production of only 3.9028 mg/g. Two types of inoculum had no significantly difference since both gives high production of ferulic acid to different substrates. The culture inoculated with mycelium plug of *Ceratocystic paradoxa* gives high titre of ferulic acid to pineapple peel meanwhile spores suspension to banana peel. Lastly, feruloly esterase activity (FEA) of each culture was also been determined in this study.

Keywords: Ferulic Acid, Pineapple Peel, Banana Peel, *Ceratocystis paradoxa*, Solid State Fermentation, Feruloly Esterase Activity (FEA), Biovanillin

ABSTRAK

Kajian ini dijalankan untuk membezakan penggunaan kulit nenas dan kulit pisang sebagai substrat dalam penghasilan asid ferulik, salah satu perintis dalam menghasilkan biovanillin melalui proses fermentasi pepejal dengan menggunakan fungus Ceratocystis paradoxa. *Kajian ini sangat penting kerana tiada lagi laporan yang mengesahkan sisi baik daripada fungus* Ceratocystis paradoxa *kerana fungus ini dikenali sebagai haiwan perosak tanaman terutamya tanaman nenas., Kajian ini juga bertujuan untuk mengenal pasti perbezaan antara dua jenis inokulum yang digunakan semasa proses fermentasi iaitu 'mycelia plug' dan 'spore suspension'. Selain itu kajian ini turut menyiasat aktiviti 'feruloly esterase untuk setiap kultur. Kajian mendapati kulit nenas menghasilkan asid ferulik yang lebih tinggi daripada kulit pisang iaitu sebanyak 5.3885mg/g manakala kulit pisang pula sebanyak 3.9028mg/g. Kajian juga mendapati tiada perbezaan yang ketara dalam penggunaan dua jenis inokulum yang berbeza kerana masing masing menghasilkan asid ferulik yang tinggi kepada dua substrat yang berlainan. Kultur yang mengandungi 'mycelia plug' menghasilkan asid ferulik yang tinggi terhadap kulit nenas manakala kultur yang mengandungi 'spore suspension' menghasilkan asid ferulik yang tinggi terhadap kulit pisang. Akhir sekali, aktiviti 'feruloly esterase' (FEA) untuk setiap kultur juga dikaji dalam kajian ini.*

Kata Kunci: *Asid Ferulik, Kulit Nenas, Kulit Pisang,* Ceratocystis paradoxa, *Fermentasi Pepejal, Aktiviti Ferulik Esterase (FEA), Biovaninilin*

CHAPTER 1

INTRODUCTION

Vanillin (4-hydroxy-3-methoxybenzaldehyde) is the main element in vanilla flavour extracted from vanilla pods and beans (Converti *et al.*, 2010). Vanillin has highly flavour compound and is widely used in the food, confectionery, perfumery, cosmetics and pharmaceutical industries (Converti *et al.*, 2010). However, natural vanillin derived from vanilla pods and beans have variable and high prices in flavourings industry market (Walton *et al.*, 2003). Meanwhile, the production of synthetic vanillin involved relatively high amount of acidic solution during neutralisation and precipitation processes and was regarded as non-natural product by the FDA and European Legislation (Zabkova *et al.*, 2006).

As stated by Cheetham, (1993) the wide difference between natural and synthetic vanillin prices, the increased demand for "natural" and "healthy" flavors have stimulated a great interest of the flavorings industry to produce natural vanillin by bioconversion from other natural sources. Therefore, as stated by Schreier (1992), a biotechnological production of vanillin, so-called biovanillin, is hoped for, which could later be classified as a natural flavour due to the utilization of natural precursors or raw materials. It is also creating much interest in biotechnological routes for flavour production from other natural sources (Priefert *et al.*, 2001). In order to make the process economically viable, it is vital to find a precursor that is chemically close to vanillin, cheap and easily available. Ferulic acid, a widely-known natural phenolic compound from lignin degradation by fungi (Knuth & Sahai, 1991) and bacteria (Betts and Dart, 1988) was deeply studied as vanillin precursor

(Rosazza *et al.*, 1995). As stipulated by *Di* Gioia *et al.* (2007), the enzymatic hydrolysis is an interesting alternative to dissolve lignin by cleavage of the ester bonds present in lignin-polysaccharide complexes (Faulds *et al.*, 2004). Free ferulic acid can be obtained from common agricultural residues, grains and beet pulp through a combination of physical and enzymatic treatments (Williamson *et al.*, 1998).

In Malaysia, pineapple and banana industries are among the earliest agro-based export-oriented industry, dating back to the 19th century. Banana and pineapple industry contribute a significant role in the country's socio-economic development. According to Malaysia Pineapple Industry Board (2011), 17,165 metric tons of canned pineapple was produced in Malaysia in which 95% of the canned pineapple is for export and the remaining 5% is for domestic consumption. As stated by Mokhtaruddin (2011), banana is mainly grown in Malaysia in a total land area of about 27,500 hectares, with Johor, Pahang, and Sarawak as the biggest banana producing states. Besides that, Mokthrarudin (2011) stated that Malaysia had increased its focus in the production of commercial varieties of banana such as the Berangan and Cavendish varieties. Table 1 shows planted area and production of banana in Malaysia from years 2008 to 2010.

Table 1: Planted area and production of banana in Malaysia
(Malaysian Agricultural Research Development Institute (MARDI), 2013)

Year	**2008**	**2009**	**2010**
Planted area (Ha)	27,418.7	27.453.5	29, 720
Prodution (Mt)	272, 330.7	279, 762.4	294,530

However, with the growing in agricultural sectors, there was a noticeable increases in the acummulation of the agricultural wastes. As reported by Omar *et al.* (2007), the volume of agricultural residues in Malaysia is in the range of 500 milions tons of year with an exponential increase of about 10%. Less than 10% of these waste are recycled, while most of them are either burnt or allowed to decompose naturally in agricultural fields or plantations (Omar *et al.*, 2007). The waste can be a serious concern since the agricultural waste materials mostly has high moisture and sugar content, thus prone to microbial spoilage and subsequently brings detrimental effects toward environment (Ong *et al.*, 2014). Moreover, decomposed of banana peels produce noxious gases such as hydrogen sulphide and ammonia that can pose serious environmental hazard (Tock *et al.*, 2010).

However, as stated by Stabnikova *et al.* (2010), many agricultural wastes could be utilized in biotechnological transformation of value-added products, such as enzymes, fuels, chemicals, and medicines. Moreover, these agricutural wastes contain beneficial elements, such as starch, sucrose, cellulose and hemicellulose (Stabnikova *et al.*, 2010).

Since pineapple and banana peel contain high amount of carbohydrates and lignocellulose which can be used as a source of carbon for microbial fermentation , it is a promising substrate for bioconversion of wastes into useful value-added products. Moreover, as reported by Tilay *et al.* (2008), Harris and Hartley (1980) pineapple and banana peels contain 0.5 to 2% extractable amount of ferulic acid, mostly in the trans-isomeric form, and esterified with the specific polysaccharides, which has the potential to act as a precursor for vanillin production.

Therefore, this research was focused to study and compare the production of biovanillin precursor which is ferulic acid from two different agro-waste; banana and pineapple peel by using plant-pathogenic organism, *Ceratocytis paradoxa* under solid state fermentation (SSF). This research was consider as significant to researcher since this was the first research employing fungus *Ceratocystis paradoxa* in production of value-added product since this fungus was known to cause black rot post-harvest disease in pineapple, banana, sugar cane and coconut (Hubert and Fourrier, 2014).

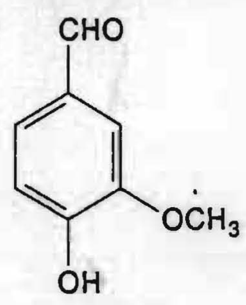
Inoculum development is one of the major unit operations in a fermentation process, involving production of required quantity of viable desired microbial biomass in its most productive state (Hockenhull, 1980). There are sucessful commercial fermentations using mycelium plugs and others use dispersed forms. In fungal solid state fermentation, the thin line between sucess and failure of a fermentation process is the quality of biomass produced as inoculum (Hockenhull, 1980). Unlike bacteria, fungal inoculum can be manipulated to required mycelium sizes or suspension forms through physical fermentation parameters to subsequently yield large quantities of the product in the main fermentation proces. Further, not many reports exists on the influence of physical parameter on the fungal morphology in inoculum development broth on the production of desired metabolites through solid state fermentation. Thus, this reseached was also aimed to investigate the effect of two type inoculum on different substrates for ferulic acid production. Two types of inoculum which were spore suspension and mycelium plug were used in this research. The difference pattern of growth and production from both types of inoculum were also been observed. Ferulolyl esterase activity was also been determined and the co-relationship of this enzyme with the production of ferulic acid were investigated.

CHAPTER 2

LITERITURE REVIEW

2.1 Vanillin

Vanillin (4-hydroxy-3-methoxybenzaldehyde) with the molecular formula, $C_8H_8O_3$ as shown in Figure 1 has the functional groups of aldehyde, ether and phenol. Nicholas *et al.* (2000) describes vanillin as an intense, sweet smell, tenacious creamy vanilla-like odour and appears as white needle-like crystalline powder. Gobley, was the first to isolate and identify the vanillin element of the vanilla bean and to confirm that this was the chief flavor component (Gobley, 1858). Besides tropical Vanilla orchid, vanillin also occurs in trace amounts in other plants. Makkar and Beeker (1994) found vanillin in tobacco meanwhile Semmelroch *et al.* (1995) found vanillin in roasted coffee and Chinese pine. Based on literature reports, commercial vanillin available in the market can be divided into two types: natural vanillin and synthetic vanillin. However, vanillin can be categorized into three types; natural vanillin, synthetic vanillin and biovanillin.



Vanillin

Figure 2.1: Chemical structure of vanillin

(Retrieved from http://commons.wikimedia.org/wiki/File:Vanillin.png

2.1.1 Types of Vanillin

2.1.1.1 Natural Vanillin

Natural Vanillin is the vanillin that isolated from the bean or vanilla pod of a tropical Vanilla orchid mainly *Vanilla planifolia, Vanilla tahitiensis and Vanilla pompona*. According to Walton *et al.* (2003) natural vanillin has very high and variable prices in the flavor industry market. The value of vanillin extracted from vanilla pods is calculated as being between \$1200 per kilo and \$4000 per kilo, compared to the price of synthetic vanillin, which is more than \$15 per kilo (Lomascolo *et al.*, 1999; Muheim and Lerch, 1999). This is due to the reason that vanilla pods being produced is inadequate to meet market demand. Furthermore, vanilla pods are reliant on soil and climate conditions. The fluctuations in crop produced also associated with the political and economic decisions, intensive cultivation, and pollination, harvesting and ripening of pods. As stipulated by Prince and Gunson (1994), natural vanillin only produces less than 1% of the total vanillin demand, with the remainder being supplied cheaply by the chemical process.

Figure 2.1.1.1(a) *Vanilla planifolia*

Figure 2.1.1.1(b) Vanilla bean

2.1.1.2 Synthetic Vanillin

According to Silva *et al.* (2005), the variable prices of natural vanillin and low supply in the market have moved development towards the chemical synthesis of vanillin. In the early 1874 to 1875, a process of synthetic vanillin production from eugenol was established and being used until the end of 1920. According to Tomlinson and Ibbert (1936), a slight vanillin odour was detected in a liquor made from sulphite pulping which was later confirmed by performing a pyrolysis of the dry residue of this liquor. This results with the idea that vanillin could be produced from lignin-rich wastes. Later, Tomlinson and Ibbert (1936) tried to begin its production from lignin-rich dry pulp but preliminary treatments were found to be too laborious. Finally, Hocking (1997) confirmed that vanillin recovered from the sulfite spent liquor comes from guaiacyl units of lignin solubilized by alkaline oxidation during softwoods pulping. Its commercial production started from an aqueous solution of the liquor containing sodium vanillate, which was purified via carbonyl sulfate by addition of H_2SO_3. However, United Kingdom and European law has not regarded chemical vanillin as a natural food component (Muheim and Lerch, 1999). Zabkova *et al.*(2006) stated synthetic vanilla production involved a relative high amount of acidic solution for neutralization and precipitation processes.

2.1.1.3 Biovanillin

Variations of prices and high market demand for natural flavors have driven flavour industry toward production of natural vanillin or so called as biovanillin from natural sources by using biotransformation (Cheetham, 1993 and Rosazza *et al.*, 1995). Biotransformation encompasses the use of enzymes or microorganisms to perform chemical reactions in which the starting substances and products are of comparable chemical complexity. Biotransformation methods are difference from biosynthesis, in which relatively complex products are assembled essentially *de novo* by whole cells, tissues, organs, or organisms from simple starting substances. In this process the substances consumedcarbon dioxide, ammonia, or glucose and those obtained from biodegradation. Biotransformation encompasses microbial transformations of organic or inorganic compounds that result in alteration in chemical structure (Walton, 1999).

2.2 Ferulic acid

Ferulic acid (FA) is the most abundant hydroxycinnamic acid found in plant cell walls. It is covalently linked by ester linkages to polysaccharides (Smith and Hartley, 1983) and ether or ester bonds to lignin (Scalbert, 1985) in plant cell walls. In nature, ferulic acid present in various plants such as citrus fruits (Swatsitang, 2000), wheat (Kim *et al.*, 2006), spinach (Fry, 1982), sugar beet (Jankovsa *et al.*, 2001), cereals (Smith and Hartley, 1983), so forth. It has many important applications in the field of medicine. Figure 2.2 summarizes all the importance of ferulic acid.

Ferulic acid was deeply studied as vanillin precursor (Rosazza *et al.*, 1995) and was predicted to be the most outstanding and potential source of natural vanillin. Research has shown that agro waste containing ferulic acid such as grains and beet pulp can be used to produce biovanillin through microbial conversion rather than conventional chemical reagents with a combination of physical and enzymatic treatments (Williamson *et al.*, 1998).

During the biotransformation of ferulic acid into vanillin, ferulic acid was released from raw materials by enzymatic treatment (Faulds *et al.*, 2004 and Williamson, 1998) and extraction (Tilay *et al.*, 2008) and then treated with various microorganisms (Lesage-Meessen *et al.*, 1996). The metabolism of ferulic acid into vanillic acid and biovanillin is reported in white rot fungi. A two-step process was described by Stentelaire *et al.* (2000) involves the transformation of ferulic acid into vanillic acid by *Aspergillus niger*, and later converted into biovanillin by *Pycnoporus cinnabarinus* or *Phanerochaete chrysosporium*.

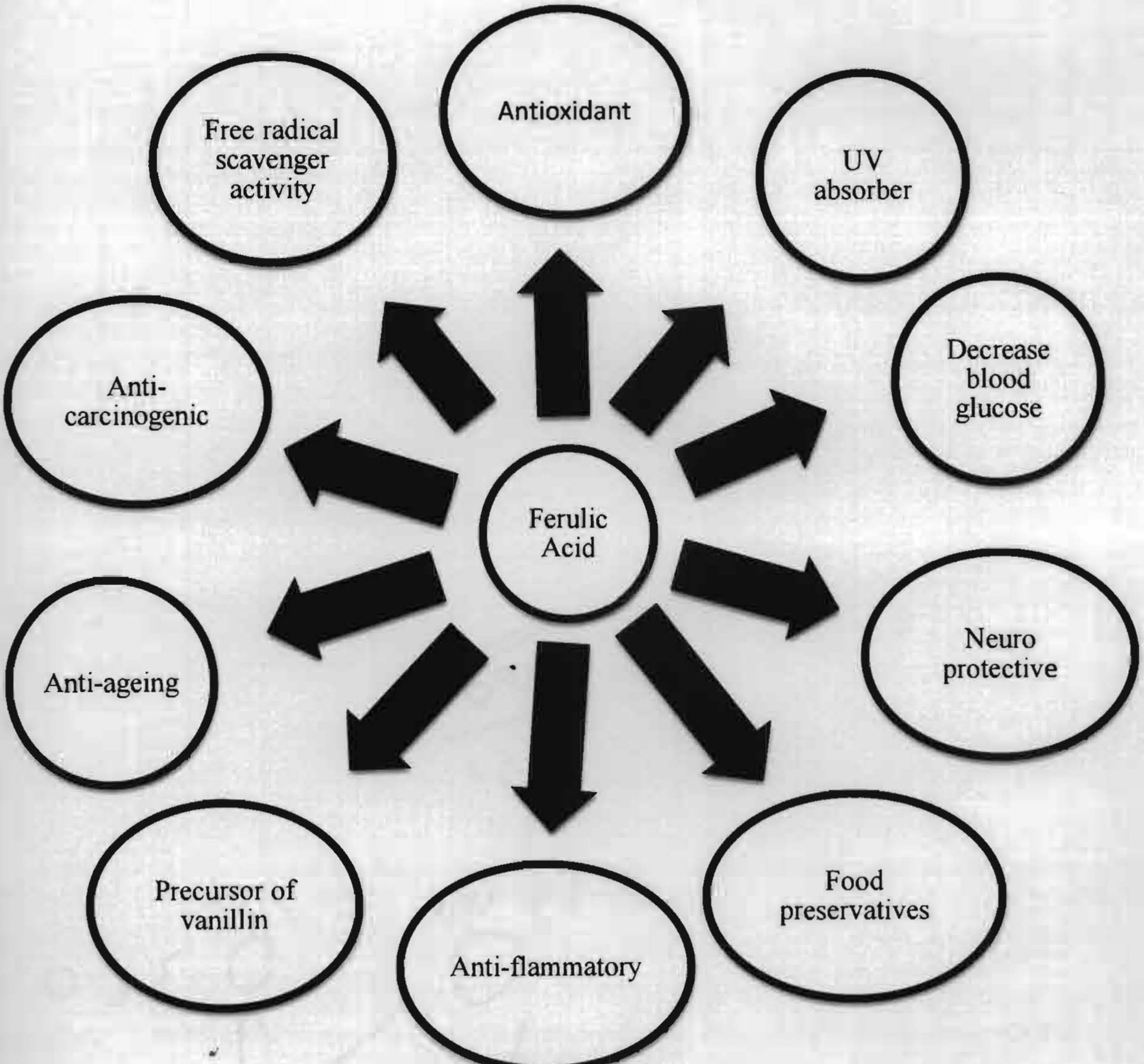


Figure 2.2.1: Applications of ferulic acid in the field of medicine (Kumar and Prithi, 2014)